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(17) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) DNA Detection Method

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Abstract

A process for the detection of a nucleic acid sequence in a homogeneous assay format using an energy transfer system is disclosed. This process utilizes a 5' labeled primer containing a selfcomplementary sequence in an amplification process together with a subsequent detection step using a 3' labeled probe for the amplified region. The labels will be close together in space after hybridizing the probe close to the short piece of double-stranded DNA resulting from backfolding of the selfcomplementary region of the primer which has been incorporated into the amplified product. The new primer for use in this process is also disclosed.

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RAN 4095/082

The present invention relates to a process for amplifying and detecting at least one specific nucleic acid sequence in a sample containing a nucleic acid or a mixture of nucleic acids at least one of which is suspected of containing said sequence, which process comprises:

5 a) amplifying the nucleic acid sequence to be detected by means of a chain extension reaction utilizing a first oligonucleotide primer of the general formula



wherein P_p is an oligonucleotide sequence substantially complementary to a part of one strand of the nucleic acid sequence to be detected, P_c is an oligonucleotide sequence substantially complementary to the sequence P_p , L is a non-nucleotidyllic linker group selected so as to allow efficient backfolding between the sequence P_c and the primer extension sequence of P_p and to avoid amplification of the backfolding part P_c , and X is an energy donor or acceptor, and a second oligonucleotide primer substantially complementary to a part of the other strand of the nucleic acid sequence to be detected;

10 b) separating after the last amplification cycle the primer extension products from their complementary sequences to produce single-stranded molecules;

15 c) treating said single-stranded molecules containing the primer of formula I above with an oligonucleotide probe of the general formula



wherein Y is an energy acceptor, when X in the primer of formula I is an energy donor, or an energy donor, when X in the primer of formula I is an energy acceptor, and P_r is an oligonucleotide sequence complementary to a part of the amplified single-stranded molecules containing the primer of formula I above and selected so as to guarantee a short distance between X and Y after backfolding of the sequence P_c and hybridization of the sequence P_r to said single-stranded molecules such that an energy transfer can take place, under conditions allowing hybridization of the sequences P_c by backfolding of P_r to said single-stranded molecules; and

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d) determining whether an energy transfer takes place.

The present invention also relates to a primer of the general formula



wherein P_p is an oligonucleotide sequence substantially complementary to a part of one strand of the nucleic acid sequence to be detected, P_c is an oligonucleotide sequence substantially complementary to the sequence P_p , L is a non-nucleotidyllic linker group selected so as to allow efficient backfolding between the sequence P_c and the primer extension sequence of P_p obtained by amplification by means of a polymerase chain reaction and to avoid amplification of the backfolding part P_c , and X is an energy donor or acceptor.

Furthermore, the invention relates to a diagnostic kit for amplifying and detecting at least one specific nucleic acid sequence in a sample containing a nucleic acid or a mixture of nucleic acids at least one of which is suspected of containing said sequence, which kit comprises a first container containing a primer of formula I as defined above and a second container containing a probe of formula II as defined above and means and reagents for amplification by means of a polymerase chain reaction and for detection.

The polymerase chain reaction (PCR) is a very powerful method for the specific amplification of DNA or RNA stretches. The methodology is described in European Patent Applications, Pub. Nos. 201.184, 200.362 and 258.017. One application of this technology is in DNA probe technology to bring up DNA present in low copy numbers to a detectable level. Numerous diagnostic and scientific applications of this method have been described by H.A. Erlich (Ed.) in PCR Technology-Principles and Applications for DNA Amplification, Stockton Press, USA, 1989 and by M.A. Innis (Ed.) in PCR Protocols, Academic Press, San Diego, USA, 1990.

A desirable goal would be the direct detection of the amplified DNA without time-consuming separations or transfer steps by a so-called homogeneous assay format. At the same time the aim is also to replace radioactive labels still mainly utilized in DNA diagnostics by nonradioactive reporter systems thereby extending the applications of this technology. Such a homogeneous detection system applying intercalating chemiluminescent acridinium esters has been reported by Arnold et al. in Clinical Chemistry 35, 1588 (1989).

Further variations of the homogeneous DNA detection assays are described in a review by E. Beckmann in *Nachr. Chem. Tech. Lab.* 37, 692-702 (1989).

The use of bathophenanthroline-Ru II complexes as nonradioactive label molecules which can be measured with high sensitivity by time-resolved fluorometry has been described by W. Bannwarth et al. in *Helv. Chim. Acta* 71, 2085-2099 (1988). These complexes can be part of an interactive pair of label molecules allowing energy transfers from suitable donor molecules onto the Ru complex. Since the efficiency of the energy transfer is highly dependent on the distance between donor and acceptor such systems can be applied in investigations of molecule interactions. As a suitable class of donor molecules for the Ru complexes lumazine chromophores have been identified. The possible applicability of this donor-acceptor pairs in the detection of e.g. DNA molecules in a homogeneous assay have been described in European Patent Application, Publ. No. 439 036, and in *Helvetica Chimica Acta* 74, 1991-1999 (1991) and 74, 2000-2008 (1991) by W. Bannwarth and F. Müller. With such a combination energy transfers were detected within oligodeoxynucleotides labeled at the 5'-end with a Ru bathophenanthroline complex and possessing lumazine chromophores at different distances from the Ru complex within the oligonucleotide. Furthermore it was demonstrated that this pair of interactive labels can also be applied to detect a target DNA sequence in a hybridization process wherein one probe sequence is equipped with the donor and the other with the acceptor.

An alternative approach using a terbium complex as an energy acceptor and salicylate as an energy donor in a homogeneous DNA detection system has been described by A. Oser and G. Valet in *Angewandte Chemie* 102, 1197-1200 (1990).

The processes known for the detection of oligonucleotides in a homogeneous test format employing energy transfers for the subsequent detection make use of at least two labeled oligonucleotides which hybridize specifically side by side to the complementary DNA sequence thereby positioning the two labels next to each other.

The basic principles of the present invention are outlined in Fig.1.

Figure 1: Shows the steps involved in an assay for amplified DNA applying interactive labels and a backfolding primer of the general formula I and a probe of the general formula II. The

complementary strand of the double strand is omitted for simplification.

In the present invention the amplification may be carried out by a combination of two primers of which at least one is a primer of the general formula I also named backfolding primer due to the ability of its subsequence P_c to fold back to the primer part P_p yielding a short double strand...

It is known in the art that selfcomplementary parts in oligonucleotides may cause problems in hybridization steps with a second oligonucleotide, especially if the first oligonucleotide is intended for use as a primer in a chain 10 extension reaction preferably with an enzyme (polymerase). Therefore, it is usually recommended for a clean reaction to optimize these primers and also probes in such a manner that they do not contain such self complementary parts.

It has now unexpectedly been found that oligonucleotides containing 15 selfcomplementary regions may be advantageously used in a polymerase chain reaction and as a detection step.

In case of the primer with formula I the primer part P_p from the 3'-end up to the nonnucleotidyl linker group represents a standard primer for the amplification of the target DNA sequence to be detected. At the temperature of 20 denaturation and subsequent utilization of a polymerase at least part of the backfolding primer exists in the open form and can thus prime specifically its template-dependent extension in the presence of adequate amounts of the four deoxyribonucleoside triphosphates (dATP, dGTP, dTTP and dCTP) or analogs. Known DNA polymerases include, for example, E. coli DNA polymerase I or 25 its Klenow fragment, T₄ DNA polymerase, Taq DNA polymerase, Tth DNA polymerase from *Thermus thermophilus* and DNA polymerase from *Thermococcus litoralis*. The reaction conditions for catalyzing DNA synthesis with these DNA polymerases are well known in the art.

Any amplification method based on a chain extension reaction may be 30 employed which allows the incorporation of the primer I in the amplification product. Amplification by the polymerase chain reaction (PCR) has already been mentioned. The primer of formula I may also be used in alternate methods of producing amplified target sequences. For example, the primer I may be used together with a second oligonucleotide which are both 35 complementary to a DNA template and are directly adjacent to one another

with their 3'- and 5'-ends. Amplification occurs through repeated denaturation, primer annealing, and ligation of the 3'- and 5'-ends. The so-called ligase chain reaction (LCR) or ligase amplification reaction (LAR) is further described by Wu and Wallace in Genomics 4, 560-569 (1988). The amplification products containing the labeled primer of formula I may then be detected by hybridizing a labeled probe of formula II near to the backfolded piece P_c as described in the next paragraphs. Amplification by PCR is the preferred method.

The denaturation temperature is about 90° - 100° C whereas the temperature for the polymerase reaction is usually lower and depends on the polymerase used. In case of the Taq polymerase the temperature is usually between 70° - 80° C which is high enough to prevent complete internal backfolding of the short sequence P_c to P_p in primer I which otherwise may inhibit the primer activity. After amplification and at low temperature the incorporated primer especially its part P_c can fold back. With a probe of formula II (detection oligonucleotide) hybridizing to the amplified region and equipped at its 3'-end with one or more energy donor molecules (if X in the primer of formula I is an energy acceptor) or an energy acceptor molecule (if X in the primer of formula I is an energy donor molecule) the backfolding guarantees a short distance between the donor or the acceptor at the 5'-end of the backfolding primer allowing in such a situation an energy transfer (Figure 1, step b)). If the target sequence is not present and therefore has not been amplified by primer I, this energy transfer is not possible since there is no primer extension product to which the detection oligomer could hybridize. Therefore a clearcut distinction between the presence of the amplified target or its absence is possible. Thus a test could be carried out directly after amplification in a homogeneous fashion without having to perform separation steps and an energy transfer indicates the presence of the amplified target sequence.

Primer I may be of any length suitable for amplification and detection. Primer region P_p has preferably a length of about 10 - 30 nucleotides but may be shorter or longer, depending on the sequence to be detected. Parameters for designing sequence and temperature specific PCR primers are known in the art taking into account the stability of the individual base pairings of the nucleotide bases.

The primer part Pp used herein is selected to be "substantially" complementary to each specific sequence to be amplified. The primer part Pp need not reflect the exact sequence of the template, but must be sufficiently complementary to selectively hybridize with their respective strands even at the temperature of polymerisation. Non complementary bases or longer sequences can be interspersed into the primer part Pp provided that this part retains sufficient complementarity with the sequence of one of the strands to be amplified to hybridize therewith, and to thereby form a duplex structure which can be extended by the polymerizing agent. Primer part Pc will be adapted in each case to be partially or completely complementary to the sequence of Pp. Preferably primer part Pp reflects the complementary sequence of the template.

The addition of the backfolding part Pc together with the label has been found to be not at all detrimental for the primer function. The sequence of Pc may have any length relative to Pp. Preferably Pc is shorter than Pp by a few bases. This precludes that, in case of a longer primer part Pc, this part binds more tightly than Pp to the complementary sequence of the template at the amplification temperature. Additional donor molecules can span the gap between 3'-end and 5'-end, which means that there is no need for the 3'-end of the probe II to start exactly where the 5'-end of the backfolded primer I ends.

Additionally, primer part Pp should not be too long in relation to Pc to avoid that the probe, at lower temperatures, hybridizes to the backfolded primer I without its prior elongation, which may cause false positive results. As an example, the primer part Pp is 18 bases in length and may fold back by 10 bases of part Pc. This is also depending on the composition of the bases in the sequence.

The second oligonucleotide primer (counter primer) commonly used for coamplification of the other (complementary) nucleic acid sequence may also be labeled and used in equal or different amounts than primer I. If both primers are labeled, almost all oligonucleotides of the amplified sequence will be labeled and may be detected using at least one 3'-labeled probe II which allows hybridization near to the backfolded part Pc of at least one of the nucleic acid sequences which have to be detected after amplification. Other variations or combinations of the primers of the invention and the probes described are within the scope of the present invention such as, for example, the use of

differently labeled primers, single strand amplification or RNA amplification instead of DNA.

Alternatively one can also place one or more of the donor molecules at the 5'-end of the backfolding oligomer and the acceptor molecules at the 3'-end of the detection oligomer as shown schematically in Figure 1, step c). Preferably the primer will be labeled with the energy donor at the 5'-end. This may be advantageous due to the background fluorescence of the energy acceptor resulting from direct excitation. In PCR the primers are applied in large excess. Thus, if the primer is labeled with the energy acceptor (e.g. the Ru complex), this fluorescence caused by direct excitation may become important.

The detection of the energy transfer between the donor and the acceptor by fluorescence measurements may be performed by methods known in the art. The methodology of the time-resolved fluorescence technique is described, for example, in the German Offenlegungsschrift No. 2628158 and in the European Patent Application, Publ. No. 178 450.

Interactive molecules may be used in any combination in the present invention, provided that they are chemically bonded or complexed to the primer or the probe without affecting the optical properties of these molecules and that they are clearly detectable in the presence of DNA. Suitable interactive molecules are glucose oxidase/peroxidase; fluorescein/mucamin and salicylate/terbium complex. The donor/acceptor combination lumazine chromophore/bathophenanthroline-ruthenium-II-complex is preferred.

The backfolding primer also contains a nonnucleotidyl group L which allows a base pairing of the complementary bases in P_c and P_p, thereby avoiding loop formation. This nonnucleotidyl group leads also to a stop of the polymerase at this position during the amplification process.

The nonnucleotidyl group L is selected so as to allow efficient backfolding between the sequence P_p and its complementary sequence P_c. This group L may consist of any kind of nonnucleotidyl linkers which allow the desired backfolding of P_c to P_p without loop formation. Preferably the nonnucleotidyl linker is derived from propanediol. More preferably the linker group L consists of two propanediol units linked together by a phosphate group and attached via phosphate groups to the oligonucleotide sequences P_c and P_p. The most preferred linker group L has the following formula:

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$$\cdots -O-P(O)_{2}^- -O-(CH_2)_3 -O-P(O)_{2}^- -O-(CH_2)_3 -O-P(O)_{2}^- -O-$$

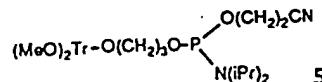
Linker group L

This nonnucleotidylic linker has been found unexpectedly to allow efficient backfolding of P_c to P_p and additionally to avoid amplification of the backfolding part P_c.

 The following description serves to illustrate some of the steps involved in the preparation of the labeled primers and probes and performing the process of the present invention.

The oligonucleotides which act as normal primers in the polymerase reaction or the oligonucleotides which are parts of the modified primers or probes can be synthesized by methods known in the art (M.J. Gait (Ed.), DNA-Synthesis - A Practical Approach, IRL-Press, 1984). Solid phase synthesis using β -cyanoethyl phosphoramidites as described by N.D. Sinha et al., in Nucleic Acids Research 12, 4539-4557 (1984) is preferred.

The group L may be inserted in the course of the solid phase synthesis of the primer part Pp with the phosphoramidite 5 or analogs thereof. The synthesis of 5 is described by F. Seela in Nucleic Acids Research 15, 3113 (1987).



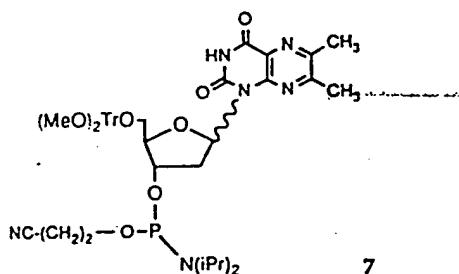
Adding two of these amidites 5 to the oligonucleotide Pp during the synthesis yields the most preferred linker which will be further elongated 20 during the synthesis with the appropriate nucleoside phosphoramidites to add the backfolding part P_c of the primer of formula I.

The labels selected for coupling at the 3'- or 5'-OH group at the end of the primer or probe may be coupled either directly or after modification of these hydroxy groups into a -NH₂, -COOH, -SH or any other suitable group with methods known in the art. The attachment of the labels may be done both if the oligonucleotide is still bonded to the support or if it is already cleaved off. The labels may also be introduced in one or more of the nucleotide bases of the primers or probes prior to their attachment to the growing oligonucleotide chain in form of, for example, nucleoside amidites. In case of Ru complex/lumazine labels the lumazine group may be introduced at the 5'-end of the backfolding primer or at the 5'-/ 3'-end of the probe using the phosphoramidite 7 of the 6,7-dimethyl lumazine-2'-deoxyriboside. Other

2090904

- 9 -

lumazine derivatives or coupling reagents for the introduction of the phosphate group may also be used.



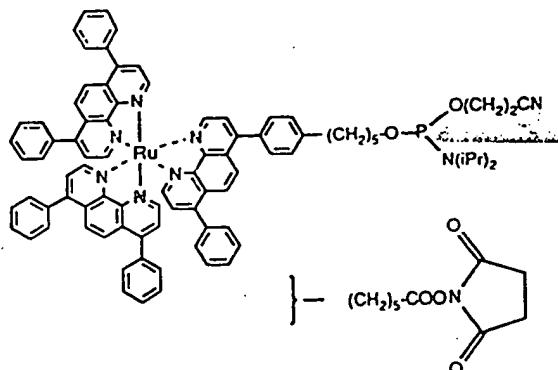
One or more of these molecules bearing the lumazine chromophore may 5 be introduced in the oligonucleotide to enhance the energy transfer. Preferred are one to four consecutive lumazine chromophores. The synthesis and introduction of the lumazine ribosides either at the 3'- or 5'-end of an oligonucleotide is described in the European Patent Application, Publ. No. 439 036.

10 Various Ru complexes as energy acceptors together with different spacers between the complex and the DNA molecule may be used as described in the European Patent Applications, Publ. Nos. 340 605 and 178 450. For the attachment of the Ru complex at the 5'-end of the backfolding primer a phosphoramidite of structure 6a or a reagent of structure 6b may be used as an 15 example. Preferably the derivative 6b may be employed for the introduction at the 3'-end.

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- 10 -

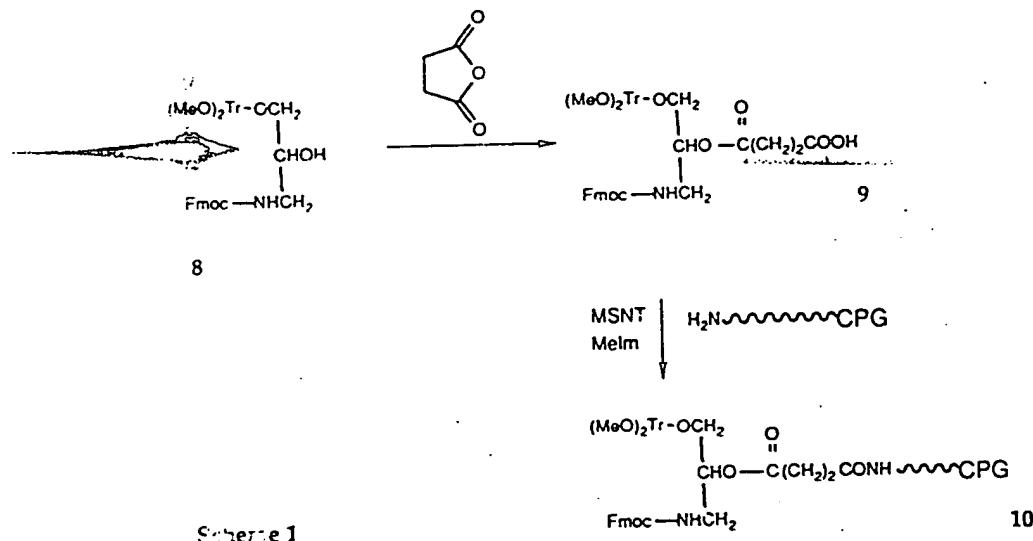
6a



6b

The reagent 6a can be used without further modifications for coupling to
5 e.g. a hydroxy or amino group in the course of the solid phase synthesis of the
oligonucleotide.

Coupling of reagent 6b at the 3'-end may be performed after some
modifications of the solid support used for the synthesis. The general scheme
is outlined below (Scheme 1) starting with compound 8 which is described by
10 Nelson et al. in Nucleic Acids Research 17, 7179 (1989). For further details
including the attachment of the Ru complex derivative 6b to the modified
support 10 see Example 2.



The invention will be further illustrated by the following examples
 5 without limiting it to them.

Example 1

Synthesis of Lumazine and Ru complex phosphoramidites

The phosphoramidite of the 6,7-dimethyl-lumazine-2'-deoxyriboside was prepared as described in European Patent Application, Publication No. 439 036 (July 31, 1991). The phosphoramidite 6a of the Ru complex was prepared in situ as described by W. Bannwarth and D. Schmidt in *Tetrahedron Letters* 30, 1513-1516 (1969).

The Ru complex derivative 6b was prepared by coupling the Ru complex with activated N-hydroxysuccinimide as described in European Patent Application, Publ. No. 349 605 or by W. Bannwarth et al., in *Helvetica Chimica Acta* 71 2085-2099 (1988). N,N,N',N'-tetramethyl (succinimido) uronium tetrafluoroborate (TSTU) was used as an activating agent. The synthesis and use of TSTU is described by R. Knorr et al., in *Tetrahedron Letters* 30, 1927-1930 (1989) and W. Bannwarth in *Tetrahedron Letters* 32, 1157-1160 (1991).

Synthesis of 1-O-(4,4'-dimethoxytrityl)-3-O-((N,N-diisopropylamino)- β -cyanoethoxy-phosphino)-1,3-propanediol 5

This reagent was prepared in a two step procedure as described by F. Seela and K. Kaiser in Nucleic Acids Research 15, 3113-3129 (1987) starting from 1,3-propanediol which was first protected by a 4,4'-dimethoxytrityl group. This was followed by a phosphorylation with (β -cyanoethoxy) bis (diisopropylamino) phosphine in the presence of diisopropylammonium tetrazolate to yield 5.

Example 2

Synthesis of 3'-Ru complex modified DNA

10 The synthesis of the modified CPG support for the solid phase synthesis of 3'-modified oligonucleotides was done as outlined in Scheme 1 (supra).

Compound 8 was prepared according to procedures described by Nelson et al., in Nucleic Acids Research 17, 7179-7186 and 7187-7194 (1989).

In the next step compound 8 (10mmol, 6.16 g) was evaporated three times from anh. pyridine. Then it was dissolved in 60 ml of anh. pyridine and 25 mmol (2.50 g) of succinic acid anhydride and 10 mmol (1.22 g) of 4-dimethylaminopyridin (DMAP) were added and stirred under argon. After 4 h the reaction was finished (TLC). The reaction mixture was taken up in 200 ml of diethylether and extracted four times with sat. brine. The organic layer was dried over Na_2SO_4 and evaporated, which yielded 6 g of an oil. Purification by short column chromatography (CC) on 150 g of silica gel (0.003-0.040 nm (Merck)) with 1000 ml of a mixture CH_2Cl_2 /MeOH/pyridine (94:5:1; v/v), 500 ml of CH_2Cl_2 /MeOH/pyridine (92:7:1) and 500 ml of CH_2Cl_2 /MeOH/pyridine (89:10:1). Pure fractions were collected and precipitated from n-pentane yielding 2.3 g of pure 9. M.p. 87-89 °C. Anal. calculated for $\text{C}_{43}\text{H}_{41}\text{NO}_9 \cdot 0.2$ n-pentane: C 72.37, H 5.99, N 1.92; found: C 72.60, H 6.14, N 1.94. $^1\text{H-NMR}$ (CDCl_3): 2.64 (s, $\text{OCCH}_2\text{CH}_2\text{CO}$); 3.03-3.50 (2m, $\text{CH}_2\text{-CH}_2$); 3.50-3.65 (m, $\text{CH}_2\text{-CH-CH}_2$); 3.74 (s, 2 OCH_3); 4.19 (t, $\text{CH}_2\text{-CH}$); 4.34 (d, $\text{CH}_2\text{-CH}$); 6.81 (d, 4 arom. H, C_6H_4); 7.15-7.37 (m, 9 arom. H, $\text{C}_6\text{H}_5\text{C}_6\text{H}_4$); 7.39 (t, fluorenyl); 7.51 (d, fluorenyl); 7.76 (d, fluorenyl).

Preparation of the functionalized support 10: The CPG-support (Pierce) was evaporated from anh. pyridine. Then it was dissolved in 10 ml of anh. pyridine and 0.60 mmol (430 mg) of 9 as well as 3 mmol (880 mg) of 1 (mesitylen-2-sulfonyl)-3-nitro-1H-1,2,4-triazole (MSNT) and 0.3 ml of

N-methyl imidazole (NMI) were added, and the suspension was allowed to react at room temperature with occasional shaking. After 2 h it was filtered off and washed successively with pyridine, DMF and ether. To the support 10 ml of a mixture of Ac_2O /pyridine (1/10; v/v) containing 1% DMAP was added.

5 After 1 h it was filtered off and the support washed with pyridine, DMF, ethanol and ether. The degree of functionalization of 10 was determined by photometric determination of the dimethoxytrityl cation (30.5 mmol/g) and after cleavage of the fluorenylmethoxycarbonyl (Fmoc) group followed by photometric determination at 300nm (30.1 mmol/g).

10 Preparation of the 3'-amino modified DNA on support 10 : The synthesis was carried out on a synthesizer starting with 1.5 mmol of support 10. A tenfold excess of the corresponding phosphoramidites was applied during each cycle. After synthesis the support was washed with acetonitrile and ether, and dried. Then 20 mg of the support were treated with 700 μl of conc. ammonia for 1.5 h at 67°C in a tightly closed Eppendorf tube. The suspension was filtered and the filtrate taken to dryness. The pellet obtained was dissolved in 300 μl of 80 % acetic acid and after 2 h 700 μl of ether were added to precipitate the DNA.

15 After centrifuging, the pellet was dissolved in water and after addition of 500 mmol of KCl it was dialysed against 2 l of water (excl. MW 1000). UV absorbancy indicated a yield of 53 OD units of amino modified DNA, which was used as such for the coupling to the Ru complex.

20 Coupling of 6b to the amino modified DNA: In an Eppendorf tube 27.5 OD units of the 3'-amino modified DNA and 4 mmol (3mg) of 6b were reacted in a mixture of 200 μl of DMF, 200 μl of dioxane, 200 μl of water and 5 μl of Hünig's base. The mixture was allowed to react for 16 h with shaking in the dark. It was taken to dryness in a speed vac concentrator and dissolved in 500 μl of water. The solution was extracted 3 times with 500 μl of CHCl_3 to remove the excess of Ru complex. The purification was performed by polyacrylamide gel electrophoresis followed by electroelution.

Synthesis of the oligonucleotides with a nonnucleotidyl linker and/or 3' or 5' modifications.

Oligonucleotides without modifications were prepared on controlled pore glass (CPG, Pierce) and applying β -cyanoethyl phosphoramidites of the appropriate building blocks. 3'-Lumazine modified oligonucleotides were

2090904

- 14 -

prepared by elongation of lumazine-2'-deoxyriboside modified CPG-support as described in European Patent Application, Publ. No. 439 036. This application describes methods and reagents for use in the synthesis of 5'-lumazine and 5'-Ru complex-modified oligonucleotides.

5 The nonnucleotidyl linker group consisting of two propanediol units separated by a phosphate group was inserted in the course of the solid phase synthesis with the corresponding phosphoramidite 5. Coupling of this amidite was performed twice, using a 10 fold excess and about 3 minutes coupling time together with tetrazole as activating agent.

10 The addition of the Ru complex at the 3'-end was performed with the Ru complex derivative 6b after the synthesis and deprotection of the oligonucleotide as described in Example 2.

Example 4

Polymerase chain reaction with a primer of the general formula I.

15 Several oligonucleotides were synthesized and tested for their ability to act as primers in the polymerase chain reaction. The DNA fragment selected for amplification is part of the gag region of HIV-1. Conventional primers with (16) or without (14,15) a Ru complex at the 5'-end as well as modified primers of the general formula I (17,18) were synthesized with methods already described.

20 The primers had the following specific sequences:

2090904

- 15 -

5'-A-T-A-A-T-C-C-C-A-C-C-T-A-T-C-C-C-A-G-T-A-G-G-A-G-A-A-A-T_{3'} 14

5'-A-T-C-C-C-A-G-T-A-G-G-A-G-A-A-A-T_{3'} 15

5'-Ru-T-A-T-C-C-C-A-G-T-A-G-G-A-G-A-A-A-T_{3'} 16

5'-T-A-T-C-C-C-A-G-T-A-G-G-A-G-A-A-A-T_{3'} 17

5'-A-T-A-G-G-G-T-C-A-T^{5'}

5'-T-A-T-C-C-C-A-G-T-A-G-G-A-G-A-A-A-T_{3'} 18

5'-A-T-A-G-G-G-T-C-A-T-Ru^{5'}

In each case the second primer or counter primer used for coamplification of the complementary strand had the following sequence:

5'-T-T-T-G-G-T-C-C-T-T-G-T-C-T-A-T-G-T-C-C-A-G-A-A-T-G-C_{3'} 19

5 These oligonucleotides were derived from primer and probe sequences already described in Table 2 of C.H. Ou et al., in *Science* 239, 295-297 (1988).

Per amplification reaction 1000 copies of HIV plasmid DNA were applied. The primers were used in excess at 100 pMoles. In case of 19 50 pMoles were applied. The HIV DNA was amplified in duplicate and a PCR negative control 10 was incorporated. Amplification with primers 14 and 19 was used as positive control.

50 µl of HIV DNA were added to 50 µl master mix for a total volume of 100 µl per reaction mixture.

Master mix:	Distilled water	27.5 µl
15	10xTaq buffer	10.0 µl
	8 mM dNTP	10.0 µl
	100 mM primer (14-18)	3.0 µl
	50 mM primer 19	3.0 µl
	Taq polymerase	0.5 µl

20 The efficiency and the homogeneity of the amplified DNA was tested by Southern hybridization (E.M. Southern, *Journal of Molecular Biology* 98, 503

2090904

- 16 -

(1975), using the radio labeled probe 5' ATC CTG GGA TTA A3'. The hybridizations were all carried out in a phosphate-buffered saline solution (10 mM inorganic phosphate; 1M NaCl, pH 7.0) with the oligonucleotides in equimolar ratio. Even the shortest backfolding primer equipped at the 5'-end with a Ru complex leads to a specific amplification comparable in efficiency with a standard nonmodified primer.

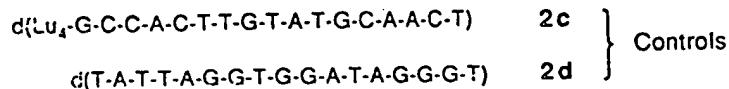
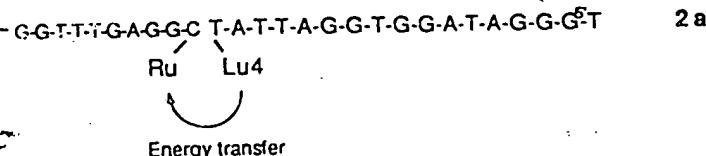
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Example 5

Detection of elongated backfolding primers via hybridization and energy transfer

10 It was determined whether a backfolding primer of the general formula I after its elongation in a polymerase chain reaction is able to serve as a template for subsequent detection with an oligonucleotide probe of the general formula II. 3'-Ru-complex-labeled oligonucleotide 1 serves as a synthetic model compound for an elongated primer of structure I which acts as a template for 3'-lumazine-labeled probes 2a, 2b and 2d and negative probe 2c.

15



(: nonnucleotidyl linker

Hybridizations were all carried out in a phosphate-buffered saline solution (10 mM inorganic phosphate; 1 M NaCl, pH 7.0) with oligonucleotides in equimolar ratio.

20 Fluorescence measurements were performed on a SLM Model 4048 S spectrofluorometer. Excitation and emission wavelengths were set at 337 nm

and 620 nm, respectively. The concentrations of the hybrids were 1.3×10^{-6} M for a sample volume of 400 μ l.

Table 1 shows the results of the energy transfer measurements. As expected, the results were positive in case of probes 2a and 2b and negative in case of probes 2c and 2d.

Hybrid	IF/IF ₂
1/2a	2.1
1/2b	2.1
1/2c	1.0
1/2d	1.2

Table 1

The fluorescence resulting from the energy transfer (IF₃) was defined as being the difference between the measured fluorescence at 620 nm (IF) minus the fluorescence of the Ru complex through direct excitation (IF₂) and the fluorescence of the lumazine chromophore at 620 nm (IF₁), which can be neglected. Therefore the formula for the intensity of the energy transfer reduces to: IF₃=IF-IF₂. For the sake of simplicity we have shown in Table 1 only the ratio of the measured fluorescence intensity IF at 620 nm in relation to the fluorescence intensity of the Ru complex due to direct excitation (IF₂).

The reversed energy transfer system utilizing a 5'-lumazine-labeled primer and a 3'-Ru complex-labeled probe was investigated with the model compounds 11, 12 and 13.

2090904

- 18 -

T-A-T-C-C-C-A-G-T-A-G-G-A-G-A-A-A-T-T-T-A-T-A-A-A-A-G-A-T-G-G-A-T-A-A₃ 11

A-T-A-G-G-G-T-C-A-T-Lu₆

Ru-T-A-A-A-T-A-T-T-T-C-T-A-C-C-T-A-T-T⁵ 12

energy transfer

T-A-T-C-C-C-A-G-T-A-G-G-A-G-A-A-A-T-T-T-A-T-A-A-A-A-G-A-T-G-G-A-T-A-A₃ 13

A-T-A-G-G-G-T-C-A-T-C-C-T-C-T-T

Ru-T-A-A-A-T-A-T-T-T-C-T-A-C-C-T-A-T-T⁵ 12

no energy transfer

The results obtained with the primer/probe systems 11/12 (showing energy transfer) and 13/12 as a negative control with no energy transfer confirmed the utility of this combination although the efficiency for the energy transfer was a bit lower (data not shown).

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A process for amplifying and detecting at least one specific nucleic acid sequence in a sample containing a nucleic acid or a mixture of nucleic acids at least one of which is suspected of containing said sequence, which process comprises:

a) amplifying the nucleic acid sequence to be detected by means of a chain extension reaction utilizing a first oligonucleotide primer of the general formula



wherein P_p is an oligonucleotide sequence substantially complementary to a part of one strand of the nucleic acid sequence to be detected, P_c is an oligonucleotide sequence substantially complementary to the sequence P_p , L is a non-nucleotidyllic linker group selected so as to allow efficient backfolding between the sequence P_c and the primer extension sequence of P_p and to avoid amplification of the backfolding part P_c , and X is an energy donor or acceptor, and a second oligonucleotide primer substantially complementary to a part of the other strand of the nucleic acid sequence to be detected;

b) separating after the last amplification cycle the primer extension products from their complementary sequences to produce single-stranded molecules;

c) treating said single-stranded molecules containing the primer of formula I above with an oligonucleotide probe of the general formula



wherein Y is an energy acceptor, when X in the primer of formula I is an energy donor, or an energy donor, when X in the primer of formula I is an energy acceptor, and P_r is an oligonucleotide sequence complementary to a part of the amplified single-stranded molecules containing the primer of formula I above and selected so as to guarantee a short distance between X and Y after backfolding of the sequence P_c and hybridization of the sequence P_r to said single-stranded molecules such that an energy transfer can take place,

2090904

under conditions allowing hybridization of the sequences P_c by backfolding and of P_p to said single-stranded molecules; and

d) determining whether an energy transfer takes place.

2. A process according to claim 1, wherein X is an energy donor and Y is an energy acceptor.

3. A process according to claim 1, wherein X is an energy acceptor and Y is an energy donor.

4. A process according to any one of claims 1 to 3, wherein the energy donor is a lumazine chromophore and the energy acceptor is a bathophenanthroline-ruthenium-II-complex.

5. A process according to any one of claims 1 to 4, wherein L consists of two propanediol units linked together by a phosphate group and attached via phosphate groups to the oligonucleotide sequences P_c and P_p .

6. A process according to any one of claims 1-5, wherein the chain extension reaction is a polymerase chain reaction.

7. A primer of the general formula



wherein P_p is an oligonucleotide sequence substantially complementary to a part of one strand of a nucleic acid sequence to be detected, P_c is an oligonucleotide sequence substantially complementary to the sequence P_p , L is a non-nucleotidyllic linker group selected so as to allow efficient backfolding between the sequence P_c and a primer extension sequence of P_p obtained by amplification using a polymerase chain reaction and to avoid amplification of the backfolding part P_c , and X is an energy donor or acceptor.

8. A primer according to claim 7, wherein X is an energy donor.

9. A primer according to claim 7, wherein X is an energy acceptor.

10. A primer according to claim 7 or 8, wherein X is a lumazine chromophore.

2090904

11. A primer according to claim 7 or 9, wherein X is a bathophenanthroline-ruthenium-II-complex.

12. A primer according to any one of claims 7 to 11, wherein L consists of two propanediol units linked together by a phosphate group and attached via phosphate groups to the oligonucleotide sequences P_c and P_p.

13. A diagnostic kit for amplifying and detecting at least one specific nucleic acid sequence in a sample containing a nucleic acid or a mixture of nucleic acids at least one of which is suspected of containing said sequence, which kit comprises a primer of the formula



wherein P_p is an oligonucleotide sequence substantially complementary to a part of one strand of the nucleic acid sequence to be detected, P_c is an oligonucleotide sequence substantially complementary to the sequence P_p, L is a non-nucleotidylic linker group selected so as to allow efficient backfolding between the sequence P_c and the primer extension sequence of P_p and to avoid amplification of the backfolding part P_c, and X is an energy donor or acceptor, and, a probe of formula II as defined in claim 1 and means and reagents for amplification and detection of said sequence using a polymerase chain reaction.

14. A nonnucleotidylic linker group derived from propanediol and containing P(O)₂⁻.

15. A nonnucleotidylic linker group of claim 14 characterized by the formula



2090904

16. A diagnostic kit for use in the amplification and detection of at least one specific nucleic acid sequence in a sample containing a nucleic acid or a mixture of nucleic acids at least one of which is suspected of containing said sequence, said kit comprising a primer of formula I as defined in any one of claims 7 to 12.

17. A diagnostic kit for use in the amplification and detection of at least one specific nucleic acid sequence in a sample containing a nucleic acid or a mixture of nucleic acids at least one of which is suspected of containing said sequence, said kit comprising:

- a) at least one of a primer of formula I as defined in any one of claims 7 to 12; and
- b) a probe of the general formula

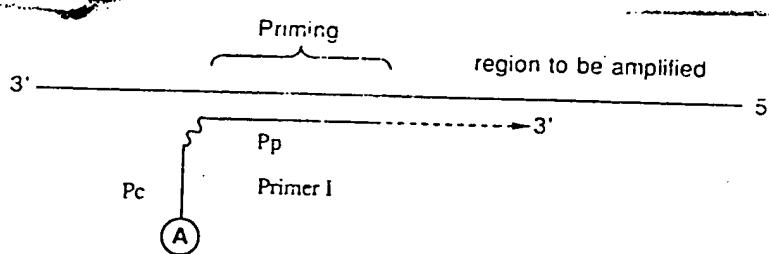
Y-Pr (II)

wherein Y is one of an energy acceptor, when X in the primer of formula I is an energy donor, and an energy donor, when X in the primer is an energy acceptor, and Pr is an oligonucleotide sequence complementary to a part of an amplified single-stranded molecule containing the primer of formula I and selected so as to guarantee a short distance between X and Y after backfolding of the sequence Rc and hybridization of the sequence Pr to said single stranded molecule such that an energy transfer can take place.

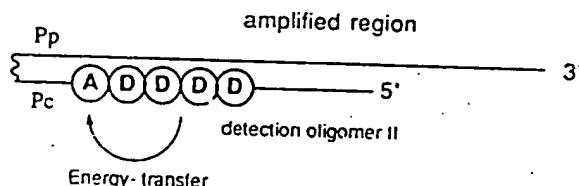
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Figure 1

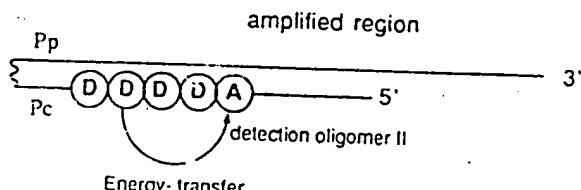
a) Denaturation and Amplification (high temperature)



b) Detection (low temperature)



c)



D : energy donor

A : energy acceptor

§ : nonnucleotidic linker L

First step